Development of a Model System for Rapid Assessment of Insect Mortality in Heated Controlled Atmosphere Quarantine Treatments

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ABSTRACT The development of postharvest quarantine treatments can be both expensive and time-consuming. It is necessary to determine the species and stage of the pest most tolerant to the treatment, if more than one species is the target of the treatment. Initial laboratory studies often include infesting the commodity with various egg and larval stages of the pest and performing treatments and evaluations of the fruit. In collaboration with others, I have previously developed combination high temperature under controlled atmosphere treatments against two quarantine pests in apples (*Malus* spp.) and peaches and nectarines (both *Prunus* spp.). I decided to develop an artificial system that can be used for these initial tests without the need for infesting large quantities of the fruit. I tested the system on the immature stages of the pests under regular air and controlled atmospheres by using the controlled atmosphere water bath system. This system can be used for rapid assessment of the most tolerant stage and species of a pest to a combination heat and controlled atmosphere treatment without the expense of infesting, treating, and evaluating the commodity.

KEY WORDS codling moth, oriental fruit moth, controlled atmospheres, heat, CATTS

Codling moth, *Cydia pomonella* (L.), and oriental fruit moth, *Grapholita molesta* (Busck), are pests of quarantine concern in pome and stone fruits exported from the United States. In the past, either fumigation with methyl bromide or the application of a systems approach has been sufficient to meet strict quarantine requirements of importing countries (NWHC 2007). However, methyl bromide fumigation has been associated with fruit damage (Neven et al. 2006), and it is not compatible with organic fruit production (NOP 2007). The systems approach is generally environmentally friendly and not associated with fruit damage; however, it can be expensive to implement in areas where pest pressures are high. The systems approach is also not accepted in some countries which require a direct postharvest treatment of commodities that may harbor actionable quarantine pests. For these reasons, high temperature treatments under a low oxygen/high carbon dioxide environment called controlled atmosphere temperature treatment system (CATTS) were developed to control these pests in apples (*Malus* spp.) and pears, peaches, nectarines, and sweet cherries (all *Prunus* spp.) (Neven 2005; Obenland et al. 2005; Neven and Rehfield-Ray 2006a, b; Neven et al. 2006).

When developing a quarantine treatment, there must be an assessment of the most tolerant stage of any insect infesting the commodity. For treatments for multiple species, the identification of the most tolerant species is required. These treatments often involve the use of infested fruits with the specific stage of a single species. These types of tests can be very expensive because it generally involves mass rearing of the target insect, obtaining fruit at the stage of maturity in which the particular stage of the insect infests, and infesting the commodity, treating the commodity, and extracting the insects from the commodity. Based on personal experience with codling moth and oriental fruit moth, I have estimated the cost for these types of experiments to run approximately $1 per insect., which includes the costs of insect production, fruit, technical labor, and treatment This may not seem expensive, considering that efficacy tests require tests with >5,000 killed and confirmation tests require tests with >30,000 killed, these treatments can become very expensive. For these reasons, we developed a system to quickly evaluate insect response to CATTS treatments without the expense of infesting, treating, and examining fruit.

The goal of this research was to determine whether an in vitro system could be used to determine the most tolerant stage of an insect pest to combination heat and controlled atmosphere treatments with results comparable to in commodity treatments.

Materials and Methods

Controlled Atmosphere Water Bath (CAWB) System. The system was composed of two water baths, two computers, gas mixing board, and an O_2/CO_2

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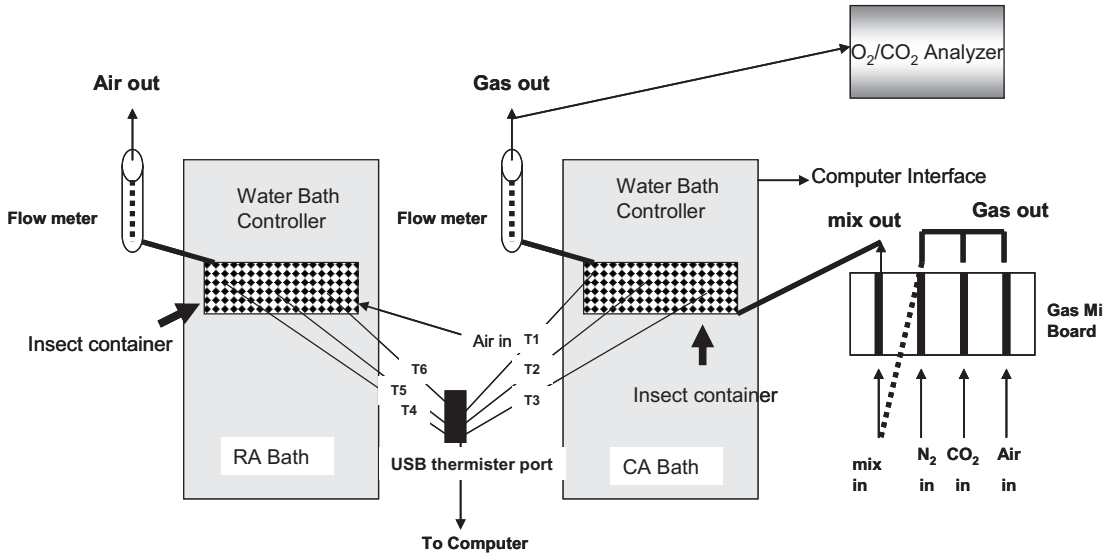

Fig. 1. Diagram of CAWB system. The system consists of two controlled water baths. The left bath has an internal controller and is used for treatments under regular atmospheres (RA Bath). The right bath is controlled by an external computer and is used for treatments under controlled atmospheres (CA Bath). The system has a gas mixing board composed of four gas flow meters. Three of the meters are connected in series and then attached to the final flow meter, which is then attached to the insect treatment container. House air is attached to the insect treatment container in the RA bath. Gas leaving the CA insect treatment container is plumbed into an O_2/CO_2 analyzer, which allows for monitoring of atmospheric gas levels during treatments. Temperatures are monitored by thermistors connected to a USB hub, with data being saved to a computer. Bath temperatures are monitored by probes T1 and T4, and randomly selected test tubes are monitored by probes T2, T3, T5, and T6. See text for further description of the system.

analyzer (Fig. 1). The water bath used in the regular air (RA) treatments was a Neslabs 110 RTE (Neslabs Instruments, Inc., defunct), which had an internal controller, independent from a computer. The water bath used in the controlled atmosphere (CA) treatments was a Neslabs 140, which contained a computer interface controller. Capacity of the RTE110 was 4 liters, and the capacity of the RTE 140 was 6 liters. The controller was connected to a lap top computer, and temperature was controlled by Neslabs program (NesCom version 2.01, Neslabs Instruments, Inc.). A gas mixing board (Dwyer 0-2.0 SCFH, Dwyer Instruments, Michigan City, IN), which controlled the inlet of house air, house nitrogen, and cylinderized carbon dioxide, combined the gases and regulated the output to the insect treatment container (described below). Gases from house air and nitrogen were regulated from the house source by a simple house valve and fine tuned with a thumbscrew valve. Cylinder CO_2 was regulated by a two-stage regulator in series with a thumb screw valve. A separate house airline was used to supply the regular air (RA) container. Flow rates in both containers were monitored by simple flow meters (0–3,000 ml/min; Gilmont Inc., St. Louis, MO). The gas composition in the CA chamber was monitored by an O_2/CO_2 analyzer (Pacific CA Systems by Techni-Systems, Chelan, WA). Humidity in the sealed containers (Fig. 2) was maintained through the use of water soaked sponges attached to the sides of the Plexiglas container. Humidity was monitored with Hobo multi-channel temperature, humidity, light monitors (Onset Inc., Pocasset, MA). Dew point was calculated by the program (HoboLITE version 2.3.0). Temperature in the water baths and test tubes was recorded by thermistors attached to a modified 5-ml pipette tip suspended above the bottom of the test tube and also attached to an USB thermistor port, which was attached to a desktop computer. The temperatures are recorded using a Thermreader program. The Thermreader application was written in VB6 by Eric Bruntjen (USDA—ARS–YARL, Wapato, WA) and interfaced, via a standard USB port, a PMD-1208LS analog interface (Measurement Computing Corporation, Norton, MA). Six 10K3MBD16 10K Thermistors (BetaTherm Corporation, Shrewsbury, MA) were read for resistance at 150-ms intervals with temperatures calculated using Steinhart-hart coefficients supplied by Betatherm Corp. Temperatures were averaged and recorded by the application according to user specifications.

Insects were confined in 10- by 160-mm glass test tubes (Fig. 3) that were secured in a Plexiglas box (Fig. 2). Insects were held in the bottom one-fourth of the test tubes by a 5-ml plastic pipette tip with the tip end cut off and covered with mite cloth (Econet 0.15 by 0.35 mm mesh; Hummert International, Earth City, MO). There were 18 test tubes in the container for the RA water bath and 28 test tubes in the container for the CA water bath. Control insects for each run were placed into a container designed for the RA water bath, but the container was not sealed and remained on the benchtop until each run was completed.

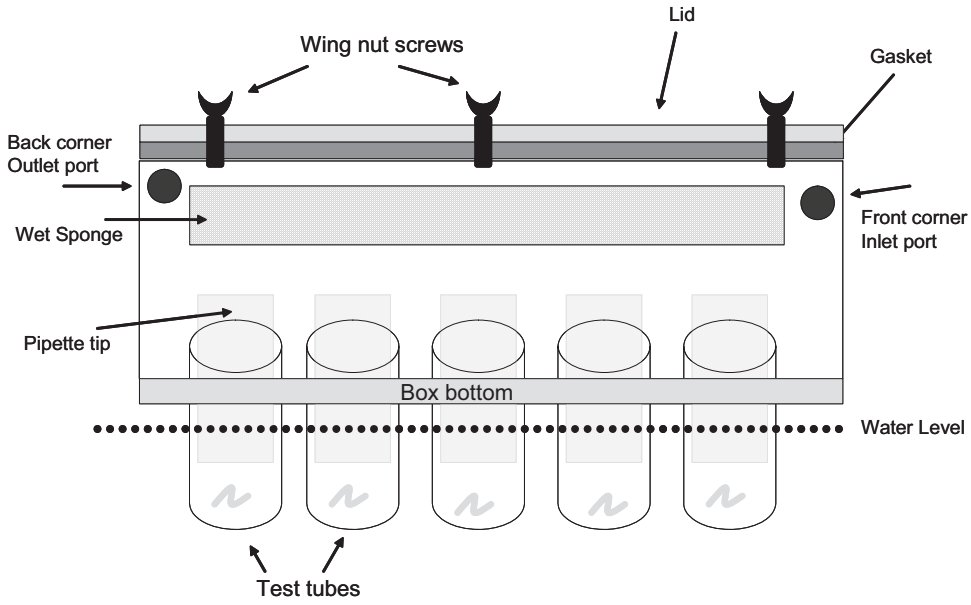

Fig. 2. Diagram of insect treatment container for CAWB. The container is composed of Plexiglas with test tubes are inserted through holes drilled into the bottom of the box and held in place with marine glue. Strips of household synthetic sponges are glued to the sides of the box to add humidification to the system. The top of the box has a compressible gasket attached to provide a gas tight seal when the lid is secured, with wing nuts, to the top of the container. There is a hole drilled through the lid to allow for insertion of temperature probes, which are held in place and sealed with marine glue. The container also has two ports on opposite sides to allow for gas injection and gas outflow. The level of water immersion is indicated by the dotted line.

Treatments. Both water baths were stabilized to 23°C before placing insects in the containers in the water. Once the temperature in both baths had stabilized, the insect containers were placed into the water and the atmosphere conditions were set. House air was used for the RA treatments with a flow rate of 1 liter/min. For the CA container, gases were mixed in the mixing board and flow into the chamber at a rate of 1 liter/min. The gas analyzer and pump were turned on to monitor O_2/CO_2 levels in the CA container. Once the levels had stabilized to 1% O_2, 15% CO_2, which normally took 5–10 min, the test was ready to begin. Oxygen levels in the chamber varied by 0.2% and carbon dioxide levels varied by 1.0% during the treatments. Both water baths were programmed to heat at a linear rate of 24°C/h to a final bath temperature of 45.5°C, which took 57.5 min, and they remained at that temperature for the duration of the test. This final bath temperature correlated to a test tube final temperature of 44.5°C. Time points were taken at 0.5, 1.0, 1.5, and 2.0 h after initiation of the heating treatment. For each time point, the treatment container was removed from the water bath, and the gases were shut off. Each time point was replicated at least four times.

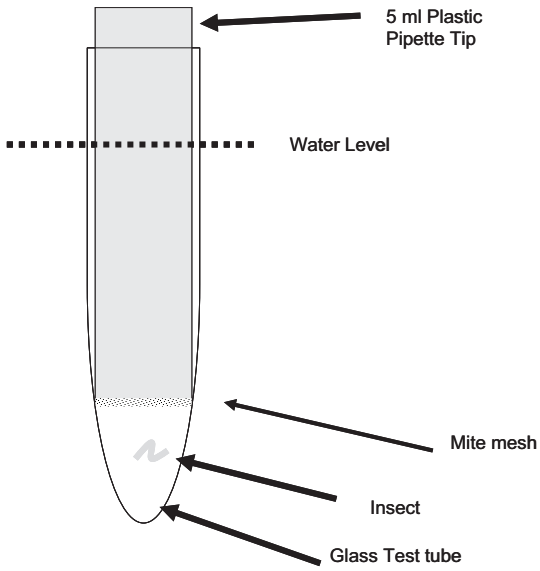

Fig. 3. Diagram of insect test tube containment setup. A 100- by 160-mm glass test tube is attached to the insect treatment container by marine glue. The insect is contained in the bottom of the test tube by a modified 5-ml plastic pipette tip. The end of the tip was cut off and fine mite mesh is attached to the heated bottom of the tube to hold it in place. The level of water immersion is indicated by the dotted line.

Insects. Both codling moth and oriental fruit moth were reared in the laboratory by using the same wheat germ-based artificial diet originally developed for codling moth (Toba and Howell 1991). Insects were reared at 23 ± 2°C, 50% RH, and a photoperiod of 16:8 (L:D) h. Eggs and first instars were collected from wax coated oviposition bags (9 by 15 by 27.5 cm, depth by width by length) containing 250 mating pairs (500 total) moths. Moths were allowed to oviposit for 24–48 h at 23 ± 2°C, 50% RH, and a photoperiod of 16:8

(L:D) h. The bag was placed at 2°C for 5 min to facilitate removal of moths. The bags were held at 23 ± 2°C, 50% RH, and a photoperiod of 16:8 (L:D) h until the desired egg stage was achieved or first instars emerged. Eggs of each desired stage were cut from the wax paper bag, counted, and placed into test tubes for a total of 30 eggs in each treatment. The number of eggs in each test tube would vary depending on the spacing of eggs on the paper. For codling moth, white ring was 0–48 h, red ring was 48–72 h, and black head was 96–120 h. For oriental fruit moth, white ring was 0–48 h, red ring was 48–72 h, and black head 72–120 h. First instars were carefully brushed from the wax paper bags using a 00 camel's-hair brush and placed into the bottom of the treatment test tube. Larvae from the second–fifth instar for codling moth and second–fourth instar for oriental fruit moth were removed from the artificial diet by hand and placed directly into treatment tubes. For each replicate, 18 larvae were used in both the untreated control and regular atmosphere treatments. Treatments under controlled atmospheres contained 28 larvae per replication. Each treatment was replicated four times for a total of 72 larvae for the controls and regular atmosphere treatments and 112 larvae for the controlled atmosphere treatments.

After treatments, eggs and larvae were removed from the test tubes and placed on organic apples that were contained in 473-ml deli containers (Reynolds Del-Pac containers, Reynolds Food Packaging and Alcoa Business, Lincolinshire, IL), and they were held at 23 ± 2°C, 50% RH, and a photoperiod of 16:8 (L:D) h. Survivorship was assessed by monitoring the eggs under a dissecting microscope for hatch 7–10 d after treatment. Survivorship in larvae was assessed 7 d after treatment. Dead larvae were collected from the bottom of the cups and sides of the apples. Live larvae were cut out of the fruit. Treatments of codling moth and oriental fruit moth larvae in nectarines are described in Neven et al. (2006).

Statistics. Data were tabulated in Excel 2000 where initial calculations on control mortality and standard error were performed. Treatment mortality was corrected for control mortality using Abbott's formula (Abbott 1925) and transformed by arcsine $\sqrt{x}$. Factorial analysis of variance (ANOVA) was performed on corrected, transformed mortalities of each species from CA and RA treatments separately using SAS version 8.2 (SAS Institute 2002). Means were separated by Tukey's honest significant difference (HSD) test. Separate ANOVA were performed on larvae and eggs because the response variables were different (mortality versus hatch).

Results

Relative humidity in the chambers indicated that the highest level of humidity was obtained at the mid-point of the run (≈1 h) and averaged 72.3 ± 0.21%, with a dew point of 67.1 ± 0.17°C for the box running the controlled atmospheres (1% O_2, 15% CO_2). The humidity in the box running under ambient

Table 1. Proportion corrected mortality ± SEM of codling moth larval stages after heat treatments in CAWB system under RA and CA environments

Time (h)	First		Second		Third		Fourth		Fifth	
	RA[a]	CA[b]	RA	CA	RA	CA	RA	CA	RA	CA
0.5	0.150 ± 0.084	0.223 ± 0.128	0.461 ± 0.176	0.362 ± 0.097	0.113 ± 0.065	0.362 ± 0.087	0.233 ± 0.134	0.460 ± 0.177	0.105 ± 0.092	0.056 ± 0.089
1.0	0.493 ± 0.103	0.978 ± 0.081	0.311 ± 0.092	0.532 ± 0.151	0.311 ± 0.193	0.532 ± 0.156	0.215 ± 0.085	0.204 ± 0.036	0.144 ± 0.101	0.290 ± 0.112
1.5	0.785 ± 0.051	0.975 ± 0.049	0.424 ± 0.049	0.672 ± 0.082	0.424 ± 0.045	0.672 ± 0.067	0.503 ± 0.035	0.934 ± 0.034	0.067 ± 0.121	0.857 ± 0.119
2.0	0.940 ± 0.138	1.00 ± 0.00	0.844 ± 0.040	1.00 ± 0.00	0.844 ± 0.049	1.00 ± 0.00	0.896 ± 0.049	0.986 ± 0.012	0.288 ± 0.140	1.00 ± 0.00

CA conditions were 1% O_2, 15% CO_2. Beginning temperature was 23°C and ending temperature was 45.5°C. Heating rate was 24°C/h. Times are total times from the start of the heat treatment.

[a] The *n* for each RA is 72.

[b] The *n* for each CA is 112.

Table 2. Proportion corrected egg hatch ± SEM of codling moth egg stages treated in CAWB system under RA and CA environments

Time (h)	White		Red ring		Black head	
	RA[a]	CA	RA	CA	RA	CA
0.5	1.00 ± 0.022	0.97 ± 0.038	0.941 ± 0.022	0.875 ± 0.044	0.956 ± 0.044	0.769 ± 0.087
1.0	0.877 ± 0.067	0.657 ± 0.128	0.908 ± 0.019	0.684 ± 0.068	0.672 ± 0.195	0.352 ± 0.155
1.5	0.781 ± 0.019	0.381 ± 0.068	0.793 ± 0.044	0.434 ± 0.091	0.574 ± 0.067	0.204 ± 0.080
2.0	0.422 ± 0.120	0.00 ± 0.00	0.300 ± 0.068	0.00 ± 0.00	0.289 ± 0.062	0.00 ± 0.00

CA conditions were 1% O_2, 15% CO_2. Beginning temperature was 23°C and ending temperature was 45.5°C. Heating rate was 24°C/h. Times are total times from the start of the heat treatment.

[a] The *n* for each RA and CA is 120.

air averaged 58.5 ± 0.18%, with a dew point of 58.5 ± 0.12°C. Relative humidity in the boxes was the lowest at the beginning of the runs, averaging 34.6% for both boxes.

The RA treatments on codling moth indicated that the fifth instar was the most thermotolerant larval stage ($F_4 = 6.72$, $P = 0.0002$) (Table 1). However, when the controlled atmosphere was applied, no instar was determined to be the most tolerant stage ($F_4 = 0.67$, $P = 0.6167$). This is in contrast to the determination of the fourth instar being the most tolerant to CATTS found for the in-fruit tests (Neven and Rehfield-Ray 2006b, Neven et al. 2006). However, the previous studies were performed at a heating rate half that used in the CAWB system, 12°C/h, and the data may not be entirely comparable.

There was no most tolerant egg stage of codling moth when the proportions of egg hatch were compared for the RA or the CA treatments ($F_2 = 1.63$, $P = 0.2134$ and $F_2 = 3.61$, $P = 0.0427$, respectively) (Table 2). This was consistent with test results from eggs treated on fruit in the CATTS chamber (Neven and Rehfield-Ray 2006b, Neven et al. 2006).

For oriental fruit moth larvae under RA treatments, the third and fourth instars were equally thermotolerant to one another and more thermotolerant than the first and second instars ($F_3 = 4.83$, $P = 0.0071$) (Table 3). Under the CA treatments, the first instar was significantly less tolerant than the second–fourth instars, who were equally tolerant to one another ($F_3 = 22.04$, $P < 0.0001$). This is in contrast with previous results obtained in in-fruit treatments, where the fourth instar was determined to be the most tolerant to CATTS (Neven and Rehfield-Ray 2006b, Neven et al. 2006). Again, the heating rate used in the CAWB was 24°C/h, whereas the in-fruit studies were carried out at 12°C/h., which may account for this difference.

Mortalities of fourth instars of codling moth and oriental fruit moth treated in nectarines (Neven et al. 2006) were compared with mortalities of fourth instars treated in the controlled atmosphere water bath (Fig. 4). Mortalities for the 1.0- and 1.5-h time points were higher in the model system than the in-fruit treatments. This may be due to increased handling of larvae in the model system and also lack of ability of the larvae to seek a slightly cooler microclimate otherwise available in the fruit.

For oriental fruit moth eggs treated under RA, no stage was determined to be more tolerant than any other stage ($F_2 = 0.27$, $P = 0.7671$) (Table 4). Under CA conditions, again, no egg stage was more tolerant of the treatment than any other stage ($F_2 = 1.65$, $P = 0.2102$). These results are in agreement with previous findings (Neven and Rehfield-Ray 2006b, Neven et al. 2006).

When the larval mortalities of both species were compared under CA conditions, neither species was significantly different from the other ($F_1 = 3.63$, $P = 0.0677$). When only fourth and fifth instars of codling moth were compared with fourth instars of oriental fruit moth, no stage or species was significantly different from the other ($F_1 = 2.92$, $P = 0.905$ for species, $F_1 = 0.38$, $P = 0.5396$ for stage). These findings are in contrast with previous research (Neven and Rehfield-Ray 2006b, Neven et al. 2006), where the fourth instar of codling moth was determined to be more tolerant than the fourth instar of oriental fruit moth.

When the response of the egg stages are compared, codling moth eggs were more tolerant of the CA ($F_1 = 162.61$, $P < 0.0001$) and RA treatments ($F_1 = 30.39$, $P <$

Table 3. Proportion corrected mortality ± SEM of oriental fruit moth larvae after heat treatments in CAWB system under RA and CA environments

Time (h)	First		Second		Third		Fourth	
	RA	CA	RA	CA	RA	CA	RA	CA
0.5	0.00 ± 0.041	0.973 ± 0.027	0.336 ± 0.140	0.259 ± 0.033	0.218 ± 0.0454	0.391 ± 0.074	0.00 ± 0.009	0.167 ± 0.012
1.0	0.905 ± 0.063	1.00 ± 0.00	0.897 ± 0.083	0.654 ± 0.019	0.351 ± 0.217	0.670 ± 0.076	0.333 ± 0.174	0.986 ± 0.009
1.5	0.911 ± 0.059	0.971 ± 0.028	0.800 ± 0.046	1.00 ± 0.00	0.814 ± 0.123	1.00 ± 0.00	0.796 ± 0.086	1.00 ± 0.00
2.0	0.882 ± 0.068	1.00 ± 0.00	0.701 ± 0.131	1.00 ± 0.00	0.707 ± 0.062	1.00 ± 0.00	0.572 ± 0.147	0.974 ± 0.026

CA conditions were 1% O_2, 15% CO_2. Beginning temperature was 23°C and ending temperature was 45.5°C. Heating rate was 24°C/h. Times are total times from the start of the heat treatment.

[a] The *n* for each RA is 72.

[b] The *n* for each CA is 112.

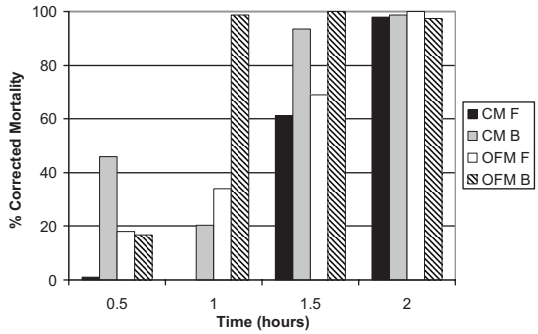

Fig. 4. Comparison of corrected percent mortalities of the fourth instars of codling moth and oriental fruit moth treated in nectarines (F) and in the controlled atmosphere water bath system. Heating rate was 24°C/h under a 1% O_2, 15% CO_2 atmosphere.

0.0001) than oriental fruit moth eggs. This is in contrast to previous studies (Neven and Rehfield-Ray 2006b, Neven et al. 2006) where the whitehead stage of oriental fruit moth was more tolerant than codling moth whitehead stage. However, the red ring and black head stages of both species were not significantly different from one another.

Discussion

In previous research, Yokoyama and Miller (1987)) determined that the that fifth instar oriental fruit moth was the most thermal-tolerant stage, with the fourth instar being slightly less tolerant. However, because our colony does not produce a fifth instar, the fourth instar of oriental fruit moth would be the most thermotolerant stage. Yokoyama et al. (1991) also determined that the fourth and fifth instars of codling moth were relatively equal in thermotolerance. In addition, comparison of thermotolerance of oriental fruit moth to codling moth (Yokoyama and Miller 1987, Yokoyama et al. 1991, Neven and Rehfield 1995) indicated that codling moth was the more thermal-tolerant species. Our data also show that codling moth fifth instars are more thermotolerant than fourth instars of either codling moth or oriental fruit moth when mortalities at the 2-h time point under RA are compared.

The CAWB system provides a good approximation of insect response to a combined heat and controlled atmosphere treatments. The CAWB can be used to identify the most thermotolerant stages (treatments under air alone) and may point the way to identifying the most tolerant stage to CATTS treatment. There is a confounding problem with CATTS in that the addition of a controlled atmosphere effectively masks stage-specific thermotolerance. We observed this phenomenon with the CAWB and also with in-fruit treatments (Neven and Rehfield-Ray 2006b, Neven et al. 2006). This may be because low oxygen environments block the production of heat shock proteins (HSPs) and ATP, critical elements of the thermal stress response in all living organisms (Neven 2003). Although differential expression of HSPs have been correlated to increased thermotolerance in different developmental stages of insects (Mahroof et al. 2005), anoxic environments seem to prevent production of HSPs in codling moth (L.G.N., unpublished), and therefore would effectively block this response.

We know from previous research that codling moth increases respiration in response to heat treatments (Neven 1998, 2000). Without sufficient oxygen to support oxidative phosphorylation, ATP levels would drastically decrease, leading to systematic cell death during a heat treatment. Low levels of ATP will also affect HSP activity. HSPs need ATP to facilitate protein folding and release (Bukau and Horwich 1998). In addition, the high levels of carbon dioxide would further acerbate the metabolic stress.

In general, terrestrial insects regulate respiration in response to internal CO_2 levels. However, in these treatments, where CO_2 levels are at 15%, normal regulatory mechanisms are inhibited. The high CO_2 levels will cause a reduction of ATP, perhaps by reducing pH through the buildup of carbonic acid (Friedlander 1983, Fleurat-Lessard 1990). In addition, low pH levels will destabilize membranes, again causing a disruption in oxidative phosphorylation and the electron transport system (Edwards 1968; Mitcham et al. 2006; Zhou et al. 2000, 2001). Cells become starved for energy, impairing their ability to stave off thermal stress damage as they would under a normal air environment.

It is evident that the application of a controlled atmosphere to a heat treatment increases mortality over that of a heat treatment under air. This is beneficial when developing heat treatments for the disinfestation of fresh horticultural commodities. This means that the addition of a controlled atmosphere will greatly shorten the heat treatment, resulting in

Table 4. Proportion corrected egg hatch ± SEM of oriental fruit moth egg stages treated in CAWB system under RA and CA environments

Time (h)	White		Red ring		Black head	
	RA	CA	RA	CA	RA	CA
0.5	0.436 ± 0.069	0.121 ± 0.038	0.430 ± 0.040	0.178 ± 0.029	0.203 ± 0.029	0.076 ± 0.033
1.0	0.360 ± 0.096	0.022 ± 0.017	0.184 ± 0.040	0.00 ± 0.00	0.302 ± 0.112	0.024 ± 0.022
1.5	0.534 ± 0.250	0.054 ± 0.022	0.472 ± 0.135	0.012 ± 0.011	0.536 ± 0.204	0.00 ± 0.00
2.0	0.303 ± 0.145	0.00 ± 0.00	0.301 ± 0.123	0.020 ± 0.009	0.251 ± 0.086	0.00 ± 0.00

CA conditions were 1% O_2, 15% CO_2. Beginning temperature was 23°C and ending temperature was 45.5°C. Heating rate was 24°C/h. Times are total times from the start of the heat treatment.

[a] The *n* for each RA and CA is 120.

less damage to the produce (Neven and Drake 2000, Neven et al. 2001, Shellie et al. 2001, Obenland et al. 2005).

The use of the CAWB system to quickly assess the most tolerant stage and most tolerant species will facilitate the development of CATTS treatments while reducing treatment development costs.

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